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A Serum Bottle Modification of the Hungate Technique for Cultivating Obligate Anaerobes

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The modification described utilizes a serum bottle closed with a butyl rubber stopper with a crimped metal seal for the growth of obligately anaerobic bacteria.

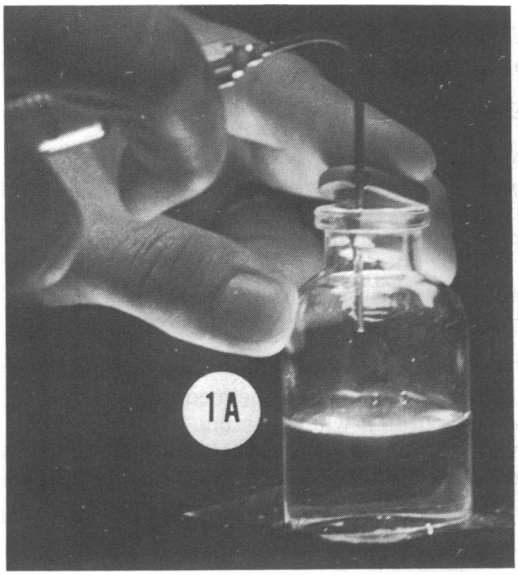
There are several derivative variations of the original technique described by Hungate for the cultivation of anaerobic microorganisms (1-4). We devised another modification which we are reporting because of its simplicity and flexibility and because of the current upsurge of interest in anaerobic methods in several areas of microbiology. The fundamental unit of operation in the modification is a serum bottle closed with a butyl rubber stopper with a crimped metal seal. Media are prepared under nonsterile conditions, usually with an appropriate reducing agent. The media are gassed with O_2-free gas and added to gassed serum bottles, and the stoppers are inserted as the bottles are withdrawn from gassing needles. Metal seals are then crimped to seal the caps to the bottles, and the bottled media are autoclaved. All inoculations are with a hypodermic syringe and needle.

The following description of the preparation of a medium for growth of *Selenomonas ruminantium* is a more detailed illustration of the method. To prepare 100 ml of medium, 0.5 g of glucose, 0.5 g of Trypticase (BBL), 0.2 g of yeast extract, 4 ml of 0.6% K_2HPO_4, 4 ml of a solution containing KH_2PO_4 (0.6%), $(NH_4)_2SO_4$ (0.6%), NaCl (1.2%), $MgSO_4 \cdot 7H_2O$ (0.245%), and $CaCl_2 \cdot 2H_2O$ (0.159%), 0.1 ml of a volatile fatty acid solution (butyric acid, 10.6 ml; isobutyric acid, 1.8 ml; 2-methylbutyric acid, 2.0 ml; valeric acid, 2.0 ml; and isovaleric acid, 2.0 ml), and 0.1 ml of a 0.1% resazurin solution are dissolved in distilled water to a final volume of 95 ml. The medium is brought to a boil and cooled rapidly in ice while being gassed vigorously with O_2-free CO_2 (3). After 5 min, 5 ml of an 8% Na_2CO_3 solution (CO_2 equilibrated) is added to the mixture. After an additional 5 min of gassing, 50 mg of cysteine-hydrochloride is added, and the complete medium is gassed continuously throughout the subsequent bottling procedure. The resazurin is usually reduced within 5 min after addition of the reducing agent. When agar is added to the medium, the agar is melted and the medium is cooled to 50 C in water while gassing with CO_2. The Na_2CO_3 solution and reducing agent are then added as described above.

Media are transferred by either a pipette or a Cornwall continuous pipetting syringe (Becton, Dickinson, and Co.) to serum bottles (Wheaton Scientific, Millville, N.J.) which are flushed with O_2-free CO_2 before, during, and after the addition of media. We found it convenient to use a manifold fitted with disposable hypodermic needles which are inserted into the openings of the bottles for flushing. A slotted butyl rubber stopper (Wheaton Scientific, catalogue no. 224154) is inserted into each bottle as the bottle is removed from the gassing needle of the manifold, with the slotted portion of the stopper facing the needle (Fig. 1A shows the procedure with a single gassing needle). The stoppered bottles are capped with tear-off aluminum seals (Wheaton Scientific, catalogue no. 224183), crimped (crimper from Pierce Chemical Co.), and sterilized by autoclaving.

All inoculations are made with disposable syringes and 26½-gauge hypodermic needles. Syringes and needles are washed with O_2-free gas by syringing from a sterile source of gas passing through an empty, sterile serum bottle. It is emphasized that in this modification, as well as in other anaerobic methods, the maintenance of anaerobic conditions necessitates the use of butyl rubber stoppers rather than stoppers made of other types of rubber. Liquid media prepared by this method has remained reduced, as indicated by resazurin reduction, during storage at room temperature for up to 6 months.

We have prepared stock slants, 4 ml of agar medium per 5-ml serum bottles (Wheaton Scientific, catalogue no. 223738), using 2-inch, 25-gauge needles to stab the butt prior to injecting 0.05 ml over the surface of the slant. When a fresh culture is needed, 0.5 ml of sterile medium is injected into the stock slant to wash off the



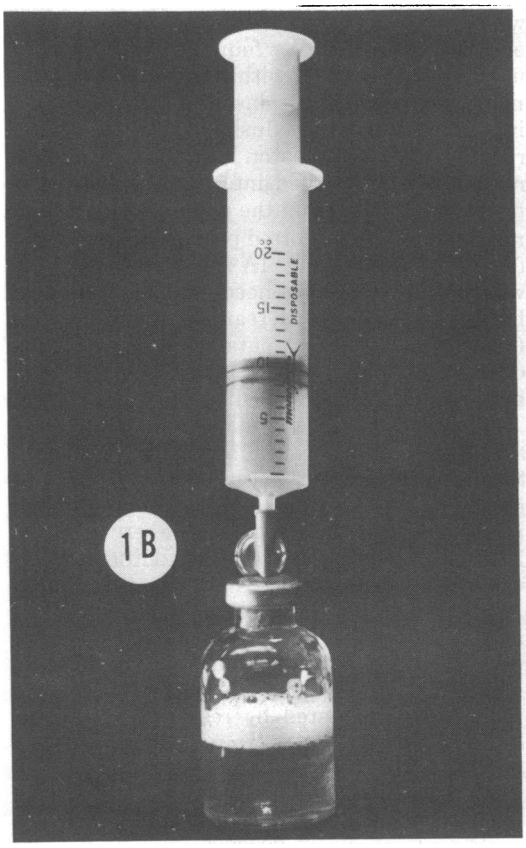


FIG. 1. *Procedures and glassware. (A) Removal of gassing needle and insertion of stopper; (B) collection of gas produced during growth.*

cells, and then 0.1 to 0.2 ml is withdrawn and injected into sterile liquid medium. We have used sterile disposable syringes fitted with 26½-gauge needles inserted into the stopper of liquid cultures to collect and measure gas produced during growth (Fig. 1B).

We have also used this method for obtaining isolated colonies. Thirty-milliliter serum bottles (Wheaton Scientific, catalogue no. 223743) containing 6 ml of melted agar medium are inoculated with 0.1 ml of the desired dilution, and roll bottles are made either manually or mechanically. Dilution blanks are prepared in the same manner as liquid media. A typical roll bottle with *S. ruminantium* colonies is shown in Fig. 2A.

The use of serum bottles for the growth of *Ruminococcus albus* has been previously described (5) In addition to *S. ruminantium* and *R. albus*, this method has been used in our laboratory to cultivate a number of nonsporeforming anaerobes, including *Ruminococcus flavefaciens*, *Bacteroides ruminicola*, and *Methanobacterium ruminantium*. In the case of *M. ruminantium*, the medium is prepared under a CO_2:H_2 atmosphere, and the reducing agent, cysteine-sulfide, is sterilized separately

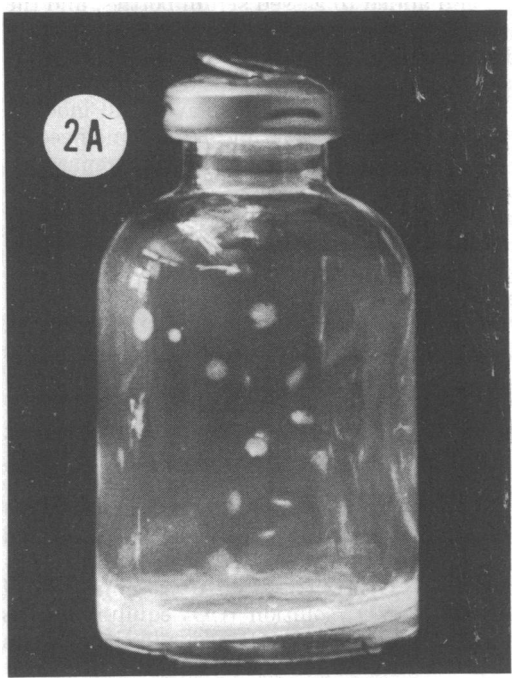


FIG. 2. *Procedures and glassware. (A) Colonies of S. ruminantium in roll bottles; (B) 18 by 150-mm test tube modified with a serum bottle neck; and (C) 2-liter bottles modified with serum bottle necks.*

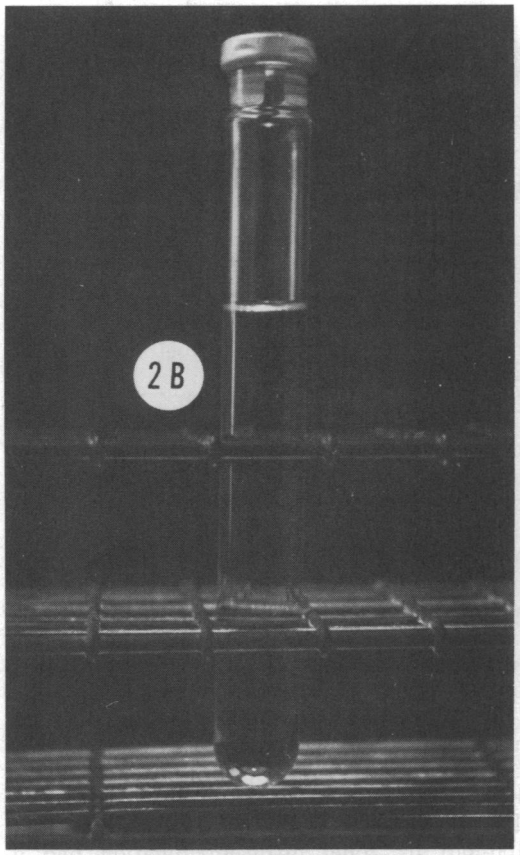


FIG. 2B

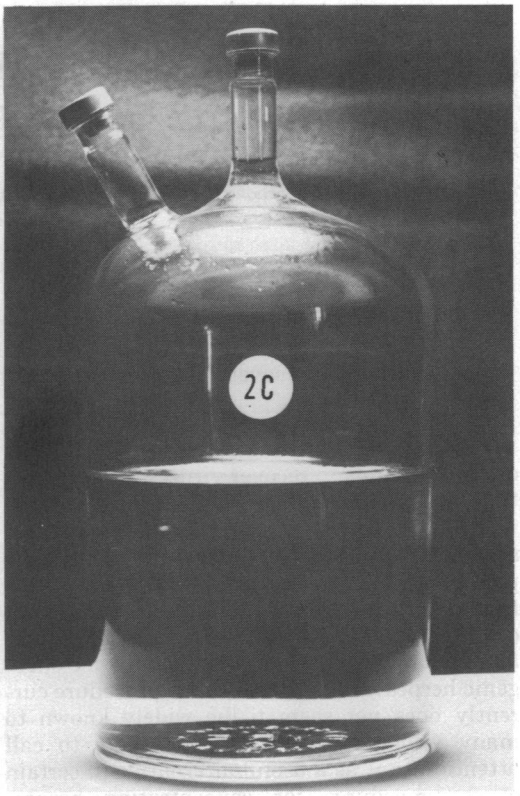


FIG. 2C

in serum bottles and added to the medium prior to inoculation.

Wheaton Scientific has prepared, according to our specifications, 18 by 150-mm test tubes with a serum bottle neck (Wheaton Scientific blueprint no. 07350) (Fig. 2B). These are convenient for studies where turbidity measurements are recorded and can be used for streaking with commercially available roll tube streaking devices (2). We have also had 2-liter Pyrex reagent bottles modified to replace the usual neck with a serum bottle neck and added a serum bottle neck sidearm (Fig. 2C). Liquid media can be prepared in these bottles, as described above, and the openings closed as with the serum bottles prior to sterilization by autoclaving. These are convenient for growing 1-liter cultures.

It is not our intention to compare the general methods outlined above with other methods for cultivating anaerobes. Our experience with various anaerobic procedures indicates to us, however, that the above method has qualities of simplicity, flexibility, reliability, and economy, which suggest its utility to others studying ecological, biochemical, clinical, and other phases of the microbiology of anaerobes, particularly those that are extremely sensitive to oxygen.

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